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HISTOCHEMICAL AND CHEMICAL STUDIES OF CALCIFEROL-INDUCED VASCULAR INJURIES

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SEVERAL extensive studies have been published on the histological aspects of vascular and other injuries inducible by calciferol intoxication (Reed, Struck and Steck, 1939 ; Ham and Lewis, 1934 ; Ham and Portuendo, 1933 ; Gillman and Gilbert, 1956*a*, *b* ; Gillman, Hathorn and Penn, 1957 ; Gillman and Hathorn, 1957). However, very little information is available about the histochemical and quantitative chemical changes in various connective tissue components, and especially in arteries, under the above circumstances.

In previous studies from this laboratory we attempted to draw attention particularly to the histological and histochemical changes induced in the connective tissues of several different arteries by acute calciferol intoxication (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; Gillman, 1957). Special emphasis was laid by us on the similarities between such injuries to blood vessels and the repair thereof, and those previously described for healing cutaneous wounds (Dunphy, Udupa and Edwards, 1956 ; Jackson, 1957). It has been, and still is our contention, backed now by far more data, that at least some forms of vascular sclerosis may be the late sequelae of intra-mural scar formation—such scars developing far more slowly in injured arteries than in cutaneous wounds (Gillman, 1957 ; 1958 ; 1959*a* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1959).

While histochemically there appeared to be very little doubt that one of the late end results of acute calciferol intoxication, namely sclerosis, was indeed the outcome of intramural scar formation, it seemed worthwhile to conduct on arteries, chemical studies of the type so successfully performed by others for healing cutaneous wounds (Dunphy *et al.*, 1956 ; Jackson, 1957 ; Grillo, Watts and Gross, 1958).

The object of the present paper is, therefore, to report the results of such a combined histological, histochemical and quantitative chemical study of the genesis of calciferol-induced vascular injuries in the rat studied from the first few days after initiation of such injuries and for a period of up to 5 months thereafter.

MATERIAL AND METHODS

Experimental.—Wistar strain rats weighing between 135–360 g. were used in the various experiments, and calciferol in arachis oil (15 mg. per ml.) was administered orally daily with a standard dropper for 5 consecutive days, as shown in Table I. All rats were weighed at

TABLE.—*Details of Experiments*

Exp. No.	Daily dose of calciferol (units)	Number of rats: Male	Number of rats: Female	Remarks
1 (*a*)	25,000	24	8	Examined histochemically at intervals up to 197 days.
(*b*)	37,000	19	—	
(*c*)	50,000	21	—	
(*d*)	75,000	3	—	
2	25,000 (1st course)	6	10	After 116 days, given 2nd course of calciferol.
	37,500 (2nd course)			Used for histochemical observations.
3	150,000 units per kg. body weight	90	—	10 calciferol-treated and 10 controls killed at 1*, 3*, 5, 7*, 10, 20, 40, 80 and 160 days. Used for chemical determinations.
	Nil (controls—pair-fed)	90	—	

* Serum Ca^{++} and SMP only.

intervals of approximately 1 week, and their clinical condition observed. In experiment 3, male rats weighing 219 ± 2 g. were used. The control rats were fed a daily quantity of food equal to that consumed by the calciferol-treated rats, until the 24th day of the experiment, when the food consumption of the latter rats equalled the *ad lib* consumption of the controls.

Histological.—The rats were killed by ether anaesthesia, the body cavities opened and the animals fixed *in toto* in 10 per cent neutral formalin. One week later the following tissues were taken and fixed in fresh 10 per cent formalin : Heart, ascending aorta and arch, thoracic aorta (longitudinal and transverse sections), abdominal aorta, kidney, stomach, colon, small bowel, spleen, mesentery, tracheal, bronchial and laryngeal cartilages and any other obviously injured tissues. Serial sections of wax embedded tissues were cut of all specimens and these were mounted on serially numbered slides. Successive slides were routinely stained by the following methods :— Weigert's resorcin-fuchsin and haematoxylin, periodic acid Schiff routine (PAS), von Kossa's method, Mallory's phosphotungstic acid haematoxylin (PAH), and alcoholic toluidine blue. Selected specimens were also recut and serial sections stained by the Rheinhart-Abul Haj ferrocyanide method for mucopolysaccharides, Wilder method for reticulin, a modified Masson method for collagen as well as several other stains including alcian blue and van Gieson's method.

Without knowledge of the nature of the treatment, the degree of injury and particularly the amount of PAS and metachromatic and ferrocyanide positive mucopolysaccharide present, collagen and von Kossa positive material was estimated by one of us (TG) according to a rough quantitative histological estimation, for each of the above mentioned findings.

Chemical.—The rats in experiment 3 were sacrificed by ether anaesthesia, and blood was obtained by cardiac puncture. Freshly excised tissues were freed from connective tissue, rinsed in saline, cut into small pieces, pooled and placed under dry acetone. After 24 hr. the acetone was replaced by fresh and allowed to stand for a further 24 hr. period. The acetone was then replaced with ether-alcohol (1 : 1) and after standing overnight the solvent was removed and the tissues dried to constant weight in a hot air oven at 100°.

Extraction of Carbohydrate and Calcium.—Portions of dry tissue (50 mg.) were heated with 4 ml. of 0·5 N hydrochloric acid for 4 hr. at 100° in sealed tubes. The solutions were then filtered and the volumes adjusted to 10 ml.

Sugar estimations.—Hexoses were determined using anthrone according to the method of Trevelyan and Harrison (1952), the results being expressed as glucose.

Amino sugars.—Hexosamines were determined by a modification of the Elson and Morgan method (1933). The acetylacetone reagent was made by dissolving 1 ml. of redistilled acetylacetone in 25 ml. of 1 M sodium carbonate (A.R.) solution. To 1 ml. of sample (containing 5–50 μg. hexosamine) was added 2 ml. of reagent and the mixture heated for exactly 15 min. in a boiling water bath. After cooling, 6 ml. of diluted PDA reagent was added, this consisting of 1·6 g. pure *p*-dimethylaminobenzaldehyde dissolved in a mixture of 30 ml. pure ethanol and 30 ml. concentrated hydrochloric acid and diluted with 300 ml. pure ethanol. The tube was allowed to stand at room temperature for 10 min. with shaking to remove CO_2 and the colour density of the solution was determined at 530 mμ. in a Zeiss spectrophotometer, model M QII, using 2 cm. cells, each run including a duplicate standard (50 μg. pure glucosamine).

Calcium.—Calcium was determined in 1 ml. samples of the acid extract (diluted with 2 ml. distilled water) by direct titration with disodium ethylene diamine tetracetic acid (·01 N), in the presence of alkali (1 ml. 2·5 N NaOH), using the calcium indicator of Diehl and Ellingboe (1956), screened with phthymolphthalein (Tucker, 1957). Serum calcium was determined by the same method.

Collagen and Elastin.—These were determined according to the method of Neuman and Logan (1950), the two 3 hr. autoclavings being replaced by single 6 hr. periods, and the urea extraction was omitted.

Serum mucoprotein.—This was determined according to the turbidimetric micro method of de la Huerga *et al.* (1956). The turbidity was read at 400 mμ in the Zeiss spectrophotometer. Armour bovine serum albumin (M.P.I.) was used for standardisation.

Apart from the phosphate and serum mucoprotein determinations, all chemical analyses were performed by one of us (RAG).

RESULTS

Clinical

After the second daily dose of calciferol, the treated rats lost their appetites and, by the 4th day of the experiment became listless ; their hair became erect, they sat hunched up in their cages and would not, as do normal rats, respond

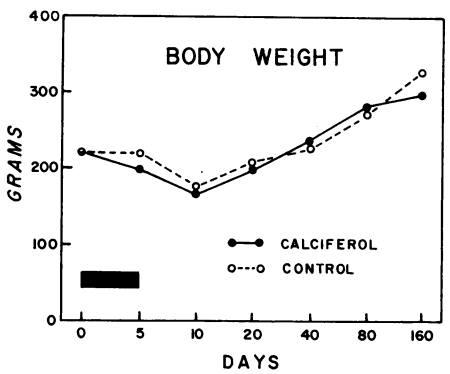


FIG. 1.—Mean body weight of rats in calciferol and control groups during course of experiment. Black bar indicates period over which the 5 daily doses of calciferol were given.

to attempts to attract them by scratching on their cages. Priapism, a recognised sign of Vitamin D intoxication (Reed, Struck and Steck, 1939) was commonly seen as was frequency of micturition and staining of the pelt with urine. Occasional rats died by the 6th day but the most severe toxic reactions and highest death rate occurred after the termination of calciferol dosing *i.e.*, by the 11th to 15th days after the first dose of calciferol.

The growth curves of the animals in Experiment 3 are shown in Fig. 1, from which it can be seen that the calciferol-treated rats lost weight rapidly during the

first 8 to 12 experimental days. They subsequently regained their initial weights by about the 30th day, whereafter they grew at about the same rate as the controls. The fact that their weight curve was almost identical to that of the pair-fed controls suggests that the weight changes were entirely due to the reduced food intake during and immediately after the calciferol administration (Fig. 2).

Macroscopic Findings

Necrosis of the spleen such as that recorded by Gillman and Gilbert (1956*a*, *b*) was encountered in many of our rats that died or were sacrificed by the 15th day. This necrotic change initially seemed to be avascular (Fig. 9) and only later was the heavily congested necrotic splenic tissue regenerated, without scar formation. There were indications that in the survivors, the damaged spleen had regenerated completely by the 25th day. Examples of macroscopic lesions consistently found in other organs are portrayed in Figs. 10 to 14.

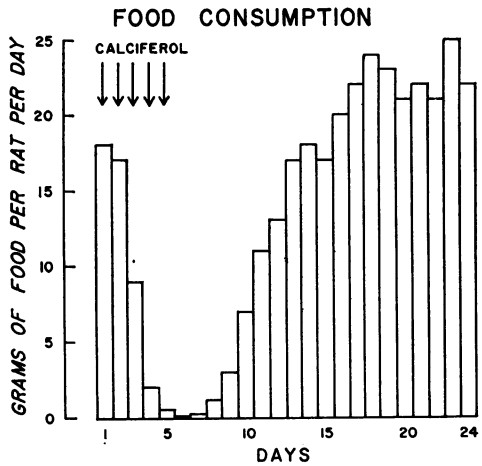


FIG. 2.—Mean daily food consumption per rat in calciferol-treated rats. The control rats, being pair-fed with the experimental rats, consumed the same daily amounts of food.

Microscopic Findings

The histological and histochemical features in rats in Exp. 1 (*b*) and 1 (*c*) were very similar, and the following description is based mainly on these two experiments. Only the lesions in the heart, coronary arteries and aorta will be described in this communication.

Heart.—The heart was severely injured very early in the experiments. The lesions were easily detectable by the basophilia of the damaged myocardium and, as shown in Figs. 15 to 18, such basophilic areas were laden with periodic acid Schiff (PAS) and von Kossa-positive material. These changes in the myocardium resolved rapidly and, by the 9th to 11th day, were replaced by highly cellular tissue diffusely infiltrating the injured areas—especially in the interventricular septum (Fig. 19). This cellularity in turn disappeared, and by the 25th day of the experiment was no longer obvious. Fibrosis of the injured myocardium was not apparent at this stage and only much later were derangements in the reticulin

pattern apparent (Fig. 20). Such late fibrosis, we believe, was not the end result of the initial necrosis but rather perhaps of some subsequent interference with myocardial integrity attendant on the coronary sclerosis and ensuing ischaemia (see below).

The myocardial necrosis, with calcification and carbohydrate accumulation, subsequent cellularity and perhaps regeneration of heart muscle (Gillman and Gilbert, 1956*b*), followed the roughly quantitative pattern outlined for von Kossa and PAS positive material shown in Fig. 3.

Coronary arteries.—It is not intended here to detail the changes in the coronary arteries encountered by us. This has, to a large extent, been done elsewhere

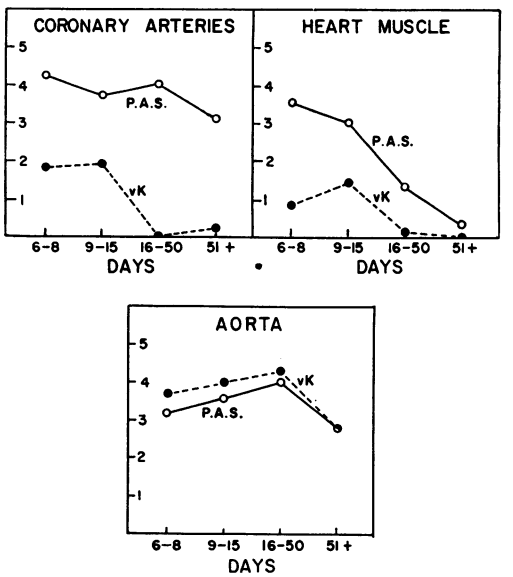


FIG. 3.—Mean arbitrary gradings of intensity of PAS and Von Kossa staining of coronary arteries, heart muscle and aorta, in rats at the indicated stages of Exp. 1.

by others and by us (Ham and Portuendo, 1933 ; Gillman and Gilbert, 1956*a*, *b* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957). However, it is important for the present study to stress the early onset of coronary dilation, injury to the internal elastic membranes, previously described, and the associated early calcification of these structures. The changes associated with repair, and the resulting sclerosis of the coronary arteries, are presented in Figs. 21 to 26. Here are shown the very early dilatation (Fig. 21), " swelling " and intense PAS-positive staining of the internal vascular elastic membranes (VEM) (Figs. 22, 23) and calcification, which resolved rapidly. Although these initial lesions are transient they are soon followed by cellularity of the adventitia and media of the affected arteries, together with both metachromatic and ferrocyanide-positive MPS accumulation (Figs. 22, 23, 24). With larger doses of calciferol, thrombosis of acutely damaged vessels was occasionally observed even in the early stages of the experiment (Fig. 24) ; this was not the case before the 40th day, however, in any of the rats on lower doses of calciferol.

The above early signs of repair were usually followed by very gradual but progressive collagenisation of the media and adventitia associated with intimal fibrin deposits, medial reticulin formation (Figs. 22, 27) and ultimately by marked stenosis with, in many instances, thrombus formation in the much narrowed lumina of the major coronary arteries (Gillman, 1957 ; Gillman and Hathorn, 1957). Marked stenosis, with thrombus formation, was almost invariable in rats in Exp. 2, i.e., those rats receiving 2 courses of calciferol. Almost all the rats treated in this manner, and studied 100 days after the second treatment, had markedly stenosed, partially thrombosed coronary arteries associated with extensive myocardial scarring (Figs. 20 and 28).

Roughly quantitative assessments of the histochemical changes seen in coronary arteries during the development of the initial lesions in Exp. 1 are presented in Fig. 3, in which the amount of early but transient, accumulations of calcium, and the persistence of carbohydrate, are clearly indicated.

Aorta.—Obvious injury, and especially calcification, with increase in perimembranous PAS-positive material, became detectable in the aorta somewhat later than in the heart or coronary arteries in some animals receiving higher doses of calciferol. However, aortic calcification and associated PAS-positive material persisted for very much longer, if not for the remainder of the life of the rats (Fig. 3).

Particularly impressive was the apparent gradient of reactivity in the arterial tree in rats receiving higher doses of calciferol. The heart and coronary vessels reacted earliest, then the ascending aorta and arch, then the thoracic aorta. Only a few days later did the abdominal aorta show the same reactions. Thus, at any particular day in the early stage of the experiments (and especially between days 3 to 10), not all parts of the aorta were equally affected, even though they became so in the experiments. This fact should be borne in mind in interpreting the chemical data which were derived from analysis of aliquots of the entire aorta, i.e., aortic arch down to aortic bifurcation.

Apart from the previously described calcification and necrosis of the tissue immediately surrounding the VEM's (the inner two to three in particular), intimal cellular reactions (Fig. 30) were often prominent (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957). Mural thrombi were also occasionally seen even in the thoracic aorta. Particularly susceptible to intimal cellularity were the aortic orifices of the coronary arteries (Fig. 31).

Cellularity of the injured aortic media itself was strikingly rare, but at 40 days necrotic and calcified medial tissue, with overlying intimal scars, was commonplace (Figs. 29 and 32). At this stage, the tissue between the fragmented calcified inner 3–4 VEM's was usually laden with metachromatic and ferrocyanide-positive mucopolysaccharide. Cartilage formation too was occasionally encountered even by the 41st day (Fig. 32). However, large plaques of heavily metachromatic cartilage were common in the aortae of rats examined at 60 to 180 days. The persistence of intense aortic calcification with cartilage formation for 100 and more days after the single period of intoxication was also a striking finding in almost all rats.

Chemical Findings

The results of estimations of serum calcium and serum mucoprotein (SMP) in the control and calciferol-treated rats in Exp. 3 are shown in Fig. 4. The mean

dry weights of the aortae are shown in Fig. 5, and the calcium, phosphorus, hexose, hexosamine, collagen and elastin content, expressed as a percentage of

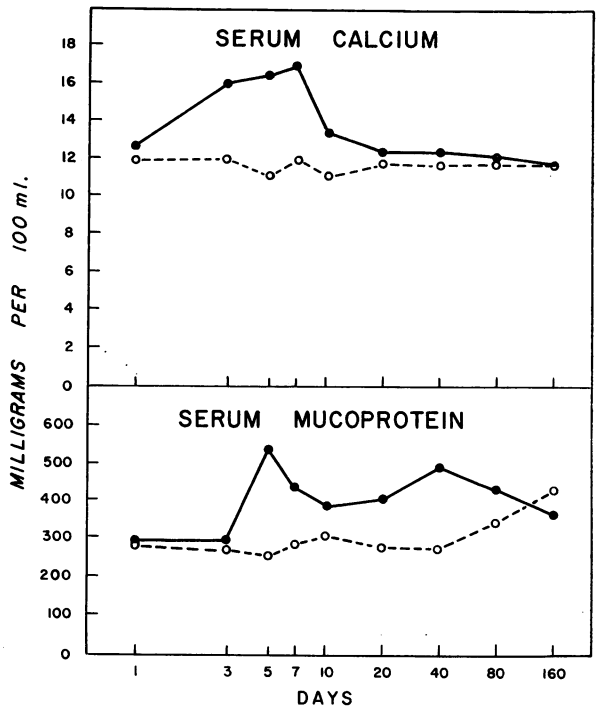


FIG. 4.—Serum calcium and serum mucoprotein in calciferol-treated (solid lines) and control (dotted lines) rats in Exp. 3.

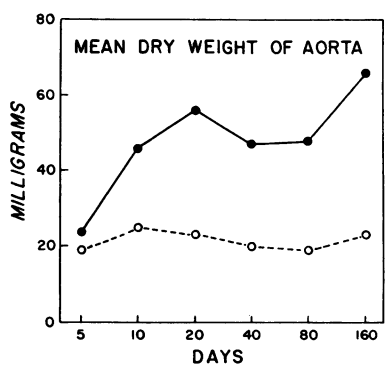


FIG. 5.—Mean dry weights of aorta in calciferol-treated (solid lines) and control (dotted lines) rats in Exp. 3.

the dry fat-free weight, are shown in Figs. 6–7. In Fig. 8 are shown the total amounts of these substances per aorta at different times after the commencement of the experiment.

The data recorded in the accompanying text figures seem to permit the following conclusions :—

The serum calcium levels (Fig. 4) rose soon after calciferol administration had been started and the levels increased until the 7th day. Thereafter, the serum calcium level dropped sharply. By the 10th day the values were only slightly above those of the controls, the latter level being reached by the 20th day of the experiment.

Comparison of Figs. 4 and 6 reveals that maximum aortic calcification occurred after the serum calcium level had fallen, and remained high until the end of the experiment. In this regard our observations confirm those of Trout (1958).

While the serum calcium level had risen sharply by the 3rd day, it should be noted that serum mucoprotein rose only on the 5th day when the serum

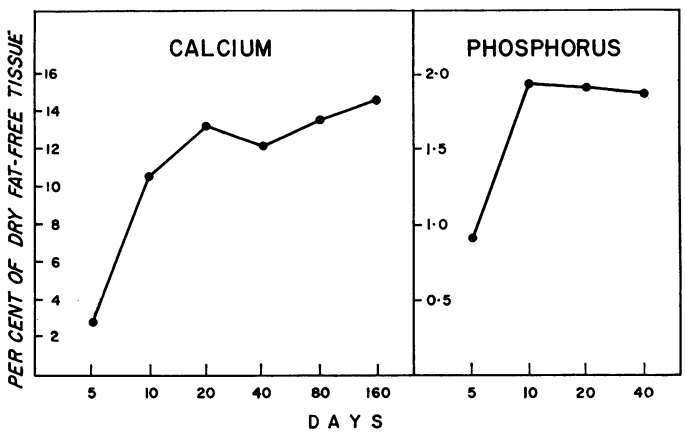


FIG. 6.—Mean calcium and phosphorus content of aorta in calciferol-treated rats in Exp. 3. The values for the control rats were too small to plot, ranging from 0·08 to 0·30 per cent for calcium, and 0·11–0·23 per cent for phosphorus.

calcium was still rising. In contrast to serum calcium, the SMP started to fall by the 5th day, but did not approach control levels until after the 80th day of the experiment, *i.e.*, long after calciferol treatment had stopped and the serum calcium level had dropped to its control level.

Some aspects of these responses in SMP will be briefly discussed below in relation to the similarities between the reactions here encountered and those described by Engel (1952) in parathormone-treated rats. Moreover, in view of the above histological data, it seems worth-while mentioning that the decreasing order of intensity of calcification of the various tissues examined by us, both chemically and histochemically was : —Aorta, lung, gastric muscle, gastric mucosa, heart, skin.

An exceptional amount of calcium accumulated in the aorta, the values in this artery approaching those in bone! (Aorta 13 to 14 per cent calcium ; femur 22 per cent calcium).

As shown in Fig. 6, calcification of the aorta was paralleled by a large increase in its phosphorus content, this heavy deposition of both calcium and phosphate resulting in a two- to three-fold increase in the mean dry weight of the aorta

above that of the controls (Fig. 5). It is noteworthy that the aortic mineralisation was dissociated from the rise in serum calcium level, for the calcification of the aorta continued after the serum calcium had fallen. These large increases in aortic mineral content were not accompanied by any change in the calcium content of the femur.

There was about a two-fold increase in the total " apparent " hexosamine content of the aortae of calciferol-treated rats, this being accompanied by an approximately parallel rise in the total hexose content (Fig. 8). These chemical changes closely parallel the histological gradings on rats in experiment 1 (*b*) (Fig. 3). It is notable too that the increases in aortic hexose and hexosamine

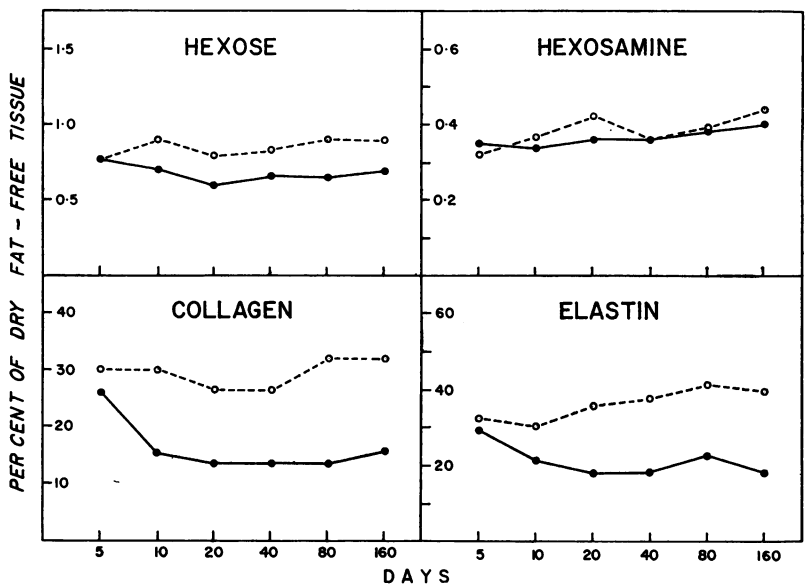


FIG. 7.—Mean percentage content of hexose, hexosamine, collagen and elastin in aortae in calciferol treated (solid lines) and control (dotted lines) rats in Exp. 3.

occurred after the marked initial rise in serum mucoprotein observed between days 5 to 7 of the experiment. However, like the SMP, so too the total aortic hexosamine and hexose values of the calciferol-treated animals once raised, remained high throughout the rest of the experiment. The increases in aortic hexose and hexosamine were, however, of much smaller magnitude than the calcium increase of about a hundred-fold. The only other component which showed a large increase similar to calcium was phosphorus (Fig. 6).

There was a large decrease in both the collagen and elastin percentages of the calcified aorta (Fig. 7). This may be accounted for by the heavy mineralization (*i.e.*, " dilution ") of elastin and collagen by calcium and phosphate.

The total collagen content of the aortae of the experimental animals (Fig. 8) had not risen significantly even by the 80th day of the experiment, a distinct increase over the control level being noted only at the end of the experiment. This chemical finding substantiates the previous report from this laboratory that

EXPLANATION OF PLATES.

FIG. 9.—Spleen of male rat, 7 days after 1st dose of calciferol, Exp. 1*d*. Note extensive areas of necrosis.

FIG. 10.—Stomach of male rat, 7 days. Exp. 1*d*. Note extensive areas of mineralization of stomach wall.

FIG. 11.—Colon of male rat, 11 days, Exp. 1*a*. Note extensive mineralization.

FIG. 12.—Heart of male rat, 7 days, Exp. 1*c*. Note areas of patchy mineralization.

FIG. 13.—Aorta of male rat, 15 days after 2nd dose of calciferol, Exp. 2. Note patchy distribution of mineral in aortic wall.

FIG. 14.—Colon of male rat, 15 days after 2nd dose of calciferol, Exp. 2. Note extensive mineralization of colon, causing intestinal obstruction, which was the cause of death in this rat.

FIG. 15.—Myocardium and commencement of aorta in male rat at 6th day of experiment, i.e., 1 day after last dose of calciferol, Exp. 1*c*. Note heavy mineralization of myocardium and large coronary artery in left of figure. Von Kossa. × 22.

FIG. 16.—Same as above, showing mineralization of larger but not of smaller coronary arteries, and of muscle immediately around these vessels. Von Kossa. × 51.

FIG. 17.—Myocardium of male rat, 7 days, Exp. 1*c*. Note mineralization of myocardium. Von Kossa. × 22.

FIG. 18.—Same field as Fig. 17. Note accumulation of PAS-positive material in regions of calcification shown in Fig. 17. PAS stain. × 22.

FIG. 19.—Interventricular septum of male rat, 10 days, Exp. 1*c*. Note marked cellular infiltration in muscle, with involvement of coronary arteries. No calcium was detected anywhere in this myocardium. Von Kossa. × 51.

FIG. 20.—Myocardium of male rat, Exp. 2, 71 days after second dose of calciferol. Note irregularity in myocardial reticulin in an area of possible old myocardial necrosis. Note also heavy reticulin around small coronary arteries in this field. Wilder stain. × 21.

FIG. 21.—Coronary artery of female rat, 7 days Exp. 2. Note dilatation of this major large coronary artery, and heavy deposition of iron in its wall at the site of calcification. Iron stain. × 48.

FIG. 22.—Coronary artery. Note minimal reticulin in highly cellular media and intima, with considerable increase in adventitial reticulin and collagen. Male rat, 41 days, Exp. 1*b*. Wilder stain. × 224.

FIG. 23.—Medium sized coronary artery from male rat, 43 days, Exp. 1*c*. Note marked thickening and PAS-positive staining of internal vascular elastic membrane, the intact endothelium, the cellular media and adventitia, which contain considerable amounts of PAS-positive material. PAS stain. × 224.

FIG. 24.—Heart of male rat, 18 days, Exp. 1*c*. Advanced stage of coronary lesion, ? early thrombosis. The coronary artery is near the bifurcation of the vessel. PAS stain. × 224.

FIG. 25.—Heart of male rat, Exp. 1*b*. Two coronary vessels with markedly thickened media and eccentric lumina. Tol. blue stain. × 66.

FIG. 26.—Heart of male rat, 41 days, Exp. 1*b*. Coronary artery to show distribution of PAS-positive material, this being in situation of a remnant of the internal vascular elastic membrane. PAS stain. × 224.

FIG. 27.—Heart of male rat, 118 days, Exp. 1*c*. Note marked increase in reticulin of media in the small vessel near the apex, which shows almost complete thrombosis. Wilder stain. × 224.

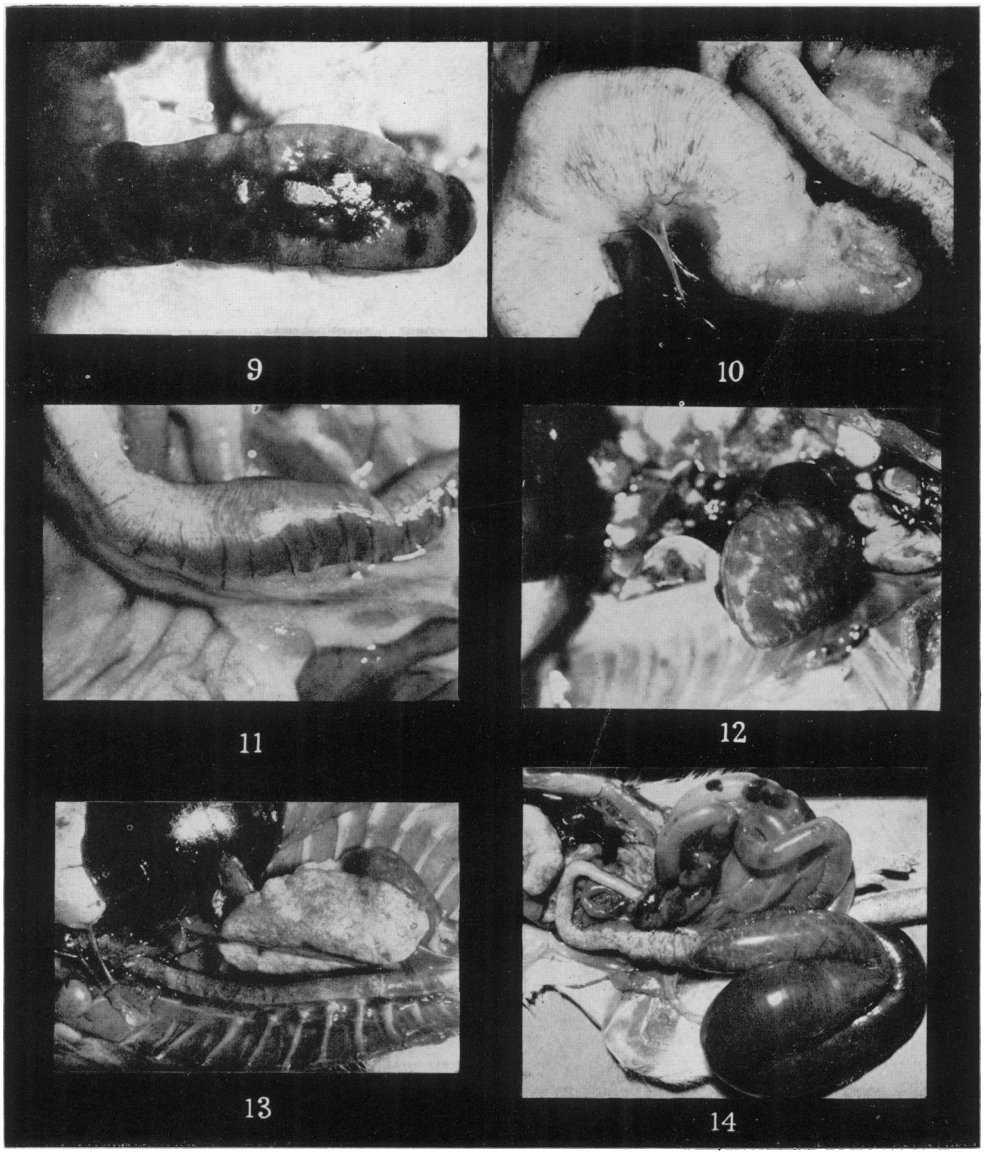
FIG. 28.—Coronary artery of male rat, Exp. 2, 71 days after second dose of calciferol. Note almost complete thrombosis, and heavy PAS-positive accumulation in wall. This reaction was very localized and not detectable in many later serial sections. PAS stain. × 120.

FIG. 29.—Aorta of male rat, 41 days, Exp. 1*b*. Note heavy calcification in area of medial damage and intimal scar. There was no calcification visible anywhere in coronary arteries or in the myocardium. Von Kossa. × 50.

FIG. 30.—Commencement of aorta of male rat, 41 days, Exp. 1*b*. Note thinning of aortic wall and marked cellular reaction in thick intimal scar with extensive thickening of aortic wall over necrotic calcified media. PAH stain. × 50.

FIG. 31.—Origin of main coronary artery of male rat, 10 days, Exp. 1*b*. Note heavy cellular infiltration, moderate endothelial lesion neighbouring mast cells; moderate metachromasia of aortic media and adventitia of coronary artery. Note also thickening of elastic membranes. Tol. blue stain. × 50.

FIG. 32.—Ascending aorta of male rat, 41 days, Exp. 1*b*. Note fragmentation of necrotic medial, PAS-coated elastic membranes; and minimal cellularity of this area of medial necrosis with early cartilage formation, overlying the intimal scar and PAS stain. × 40.



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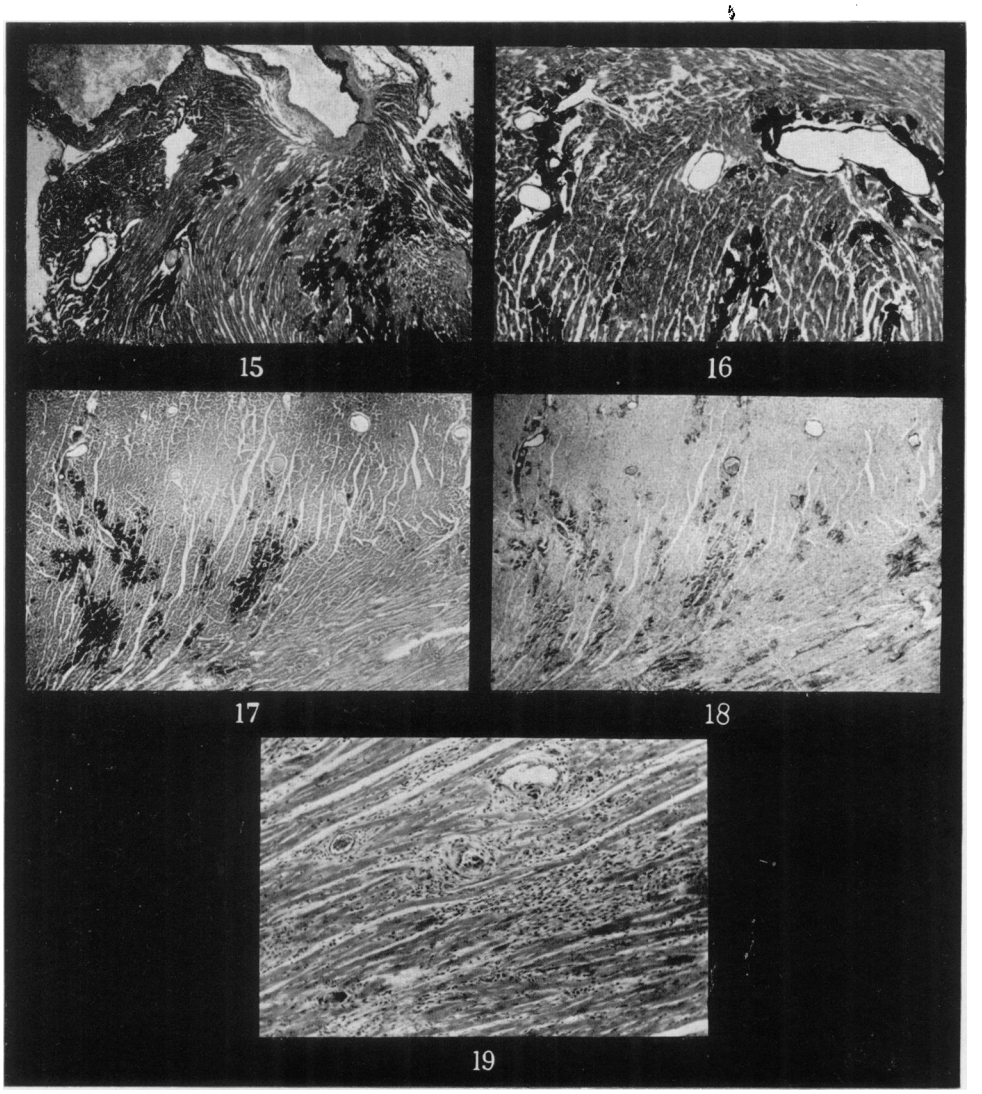
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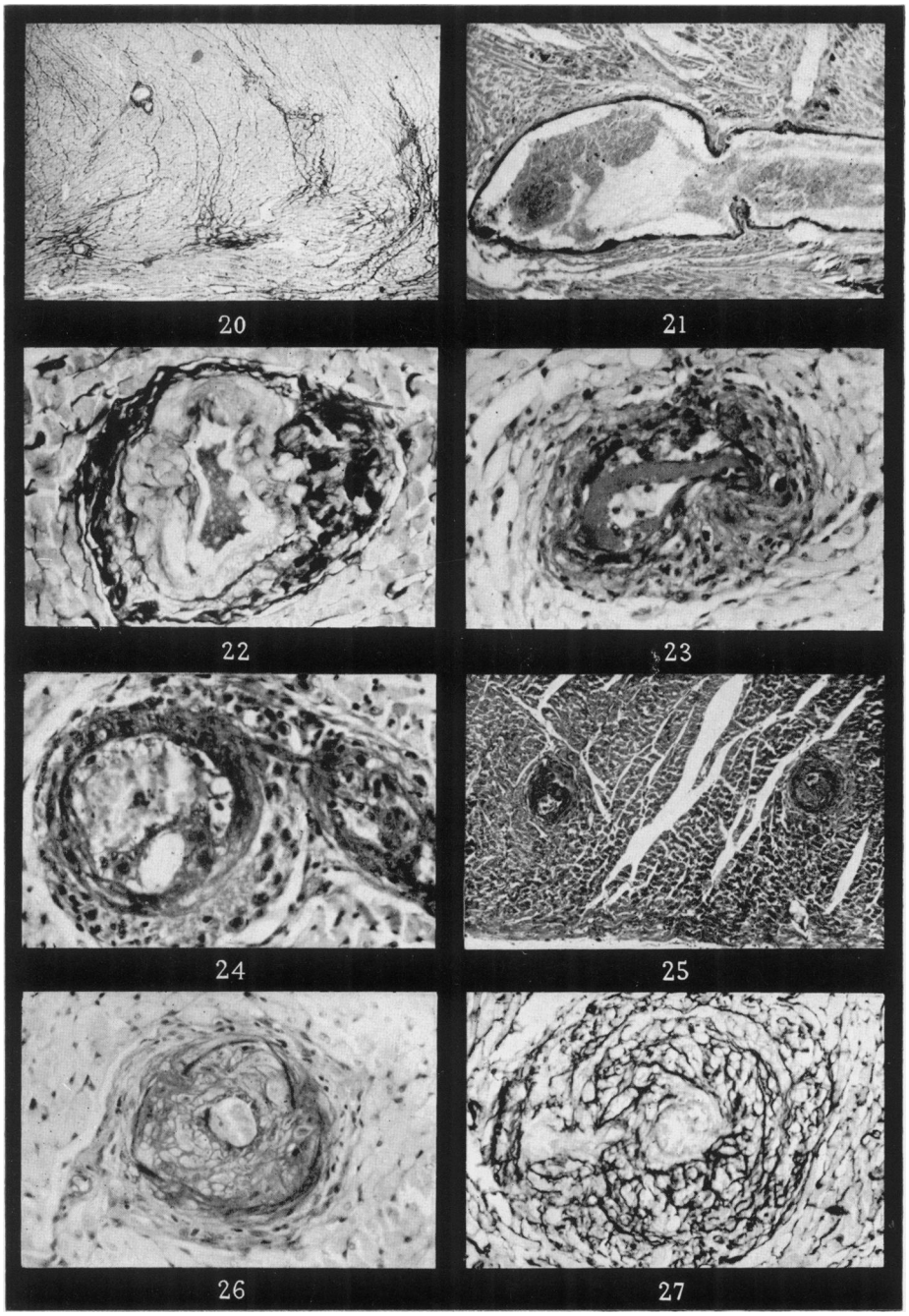

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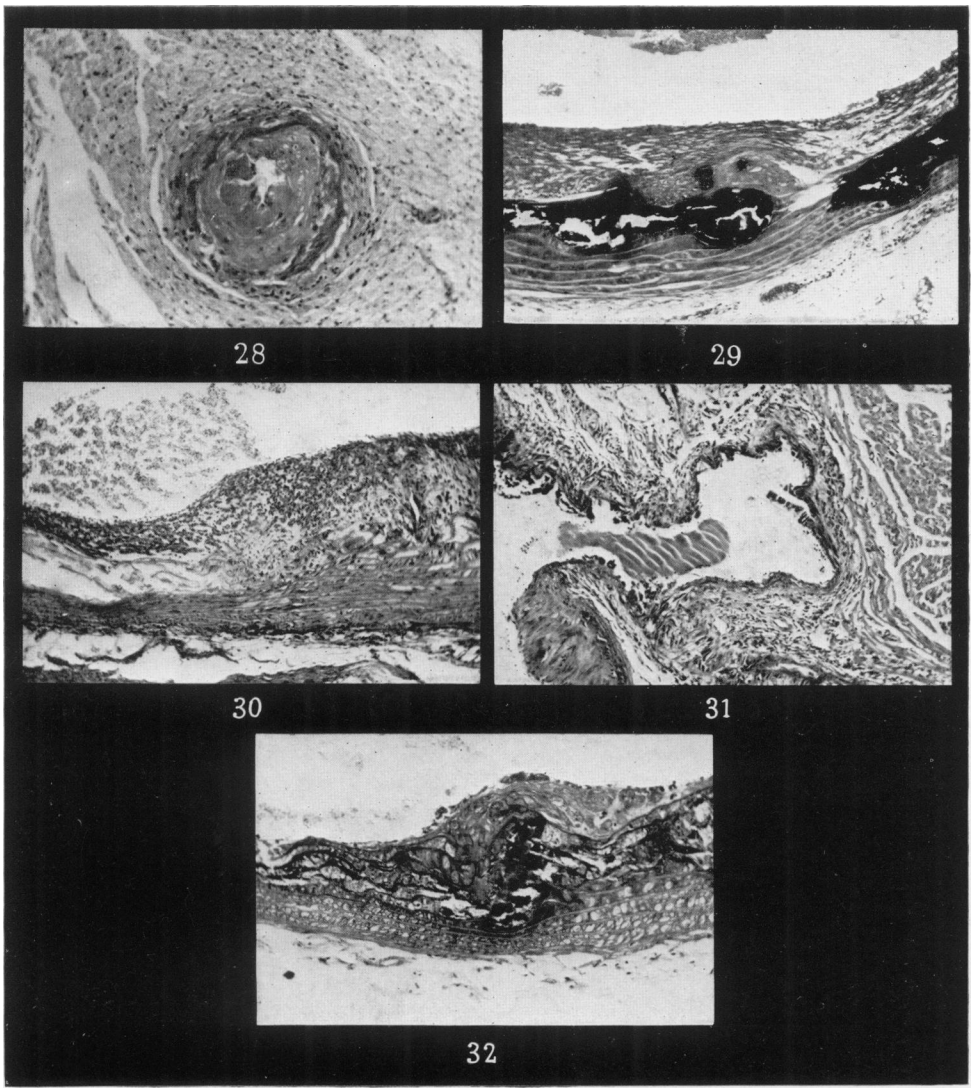

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collagenisation of severely injured vessels takes place far more slowly than is the case in healing cutaneous injuries (Gillman and Hathorn, 1957).

Among the most puzzling of our chemical findings was the change in the total " apparent " elastin (Fig. 8). It will be noted that, compared with the controls, the percentage of " apparent " elastin of the experimental group seemed to decrease in the same way as did the percentage of aortic collagen (Fig. 7). However, as indicated for collagen, this seeming decrease is probably attributable to " dilution " of the connective tissue component by the mineralisation of the aorta. The total " apparent " elastin content, however, was somewhat elevated in the calciferol series during the whole of the experiment (Fig. 16).

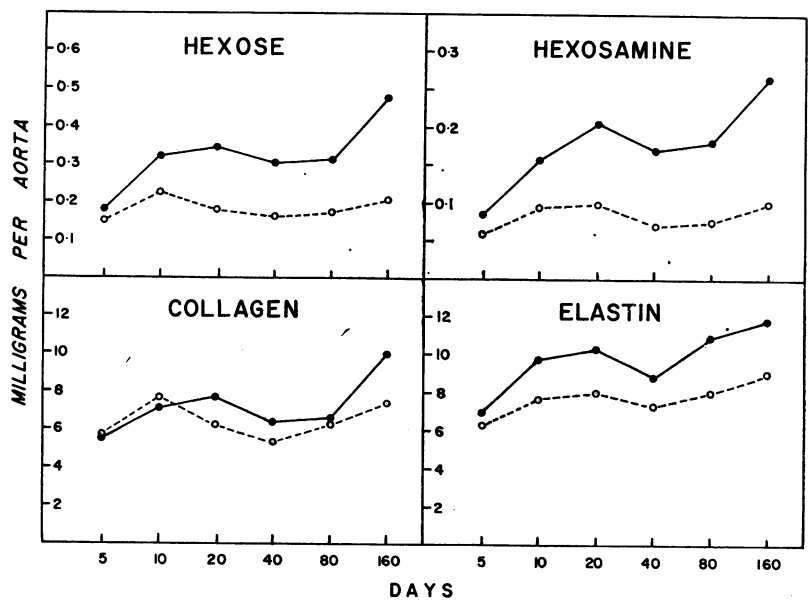

FIG. 8.—Absolute content of hexose, hexosamine, collagen and elastin in aortae in calciferol treated (solid lines) and control (dotted lines) rats in Exp. 3.

Histologically, there was no detectable increase in either elastic or " pseudo-elastic " fibres (the latter defined elsewhere—Gillman *et al.*, 1955). In fact, fractures and fragmentation of the original elastic membranes, consequent on perimembranous mineralization, were common. Such fractures were later followed by marked localised fibrotic reactions, and as already recorded elsewhere, no obvious regeneration of the vascular elastic membranes was noted at any time, even 300 or more days after a single period of calciferol intoxication (Gillman *et al.*, 1957 ; Gillman, 1957 ; 1958 ; 1959*a*). In view of the absence of any histologically demonstrable increase in elastic fibres, we considered the possibility that the apparent increase in chemically estimated elastin may have been partly attributable to the Weigert- and orcein-positive material easily demonstrable within the cartilage plaques which developed in the aortic media. Our initial studies of rat costal cartilages have revealed a small amount of hydroxyproline which is resistant to autoclaving and which may account for the apparent increase in chemically determined elastin.

DISCUSSION

The primary aims of the present combined histological, histochemical and quantitative chemical analyses of the changes in the heart, coronary arteries and the aortae of calciferol-intoxicated rats were to determine :—

(*a*) Whether the microscopic findings were indeed providing a reliable indication of the chemically determinable changes, particularly in the aorta. If the roughly quantitative microscopic assessments of the changes in the aortae are trustworthy, then we assume that such data will be acceptable for other tissues. The comparative chemical and histochemical findings in other tissues, will, however, be discussed and assessed by us in future publications.

(*b*) Whether the sequence of events in severely injured vessels, as delineated microscopically (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1958) could be confirmed by quantitative chemical analyses.

We consider that the data here provided strongly indicate that the previously described microscopically determined changes, particularly in the aorta, are fully supported and somewhat amplified by the present chemical findings.

Perhaps of special interest, in the light of recent work, are our observations on the serum mucoprotein changes in our calciferol-intoxicated rats. For Faber (1949) reported a progressive increase in chemically estimated human aortic mucopolysaccharides, with ageing, and especially in association with cholesterosis. This finding conforms with the histochemical observations of Moon and Rinehart (1952), Taylor (1953) and Moon (1957). More recently, Noble *et al.*, (1957) reported increases in aortic hexosamine with ageing. In conformity with the observations of the histologists quoted above, Noble and associates opined that such increases in aortic carbohydrates and mucoproteins comprised the earliest indications of aortic degeneration.

Schwartz and Gilmore (1958) have also recently related the serum mucoprotein and hexosamine levels in man to the existence of atherosclerosis in living subjects, and they noted elevations of these plasma fractions in ageing individuals. These workers attributed the increase in circulating SMP and hexosamine to depolymerisation of aortic connective tissue ground substance—a change which they considered to play an important role in the pathogenesis of human atherosclerosis.

Certain stimuli are known to produce elevations in SMP in experimental animals and in man. In particular, Engel (1952) showed in rats, that injections of parathormone were rapidly followed by a rise in SMP ; this he attributed to depolymerisation of the ground substance of the bones. Bloomfield and Hayes (1952), in addition, showed similar mobilisation of ground substance from cutaneous connective tissue inducible by parathormone. Kushner *et al.* (1956) demonstrated that in man, too, parathyroid extracts were among the most potent substances tested by them in elevating serum mucoproteins, although they found that fever and intensive ACTH therapy could also exert similar effects.

As shown above, in our califcerol treated rats SMP values rose only several days after the first dose and only after the observed elevation of the serum calcium levels. These two findings may have been closely related, as Engel claimed for his parathormone-treated rats. On the other hand, histological studies revealed in our rats a widespread appearance of PAS-positive material in arterial walls simultaneously with calcification. The latter may have been due, in part at least,

to the increased avidity for calcium of these newly appearing carbohydrates in the calcifying tissues. For Reaven (1953) and Sobel (1955) have averred that metastatic calcification was facilitated by alterations and especially by any increase or depolymerisation of tissue mucoproteins. This may well be the case for our rats especially since metastatic calcium deposits in aortae, coronary and other arteries, gastric muscle and mucosa, kidneys etc. were consistently associated with localised increases in the PAS-positive material located in precisely the same sites as calcium deposition (compare Figs. 15–18; also Gillman *et al.*, 1957).

Which of the above two changes came first, however, we could not ascertain with confidence. However, we do believe that alterations in the connective tissue ground substances, in all the above sites, probably played some role in the localised binding of calcium in injured areas. However, tracheal, bronchial and other cartilages, which are always laden with mucopolysaccharides even in normal rats, fail to calcify early. Also impressive was the rapid disappearance of calcium from the heart muscle, coronary arteries, gastric mucosa, pulmonary alveolar wall, renal tubules and their basement membranes—sites which are usually calcified at one phase of the initial responses to calciferol but in which PAS-positive material persists after calcification has resolved. While minerals undoubtedly accumulated in polysaccharide-laden sites in all the above tissues, the same minerals equally could, and usually did, disappear later, even though the muco- and other polysaccharides were still present in increased amount in the injured tissues.

It therefore seems that the presence of MPS is not alone effective in promoting calcification of tissues, although these mucopolysaccharides may, under certain circumstances, perhaps facilitate the binding, not only of calcium, but also of iron and especially of fat (Gillman and Naidoo, 1957; Gillman, 1957; Gillman *et al.*, 1957; Gillman and Hathorn, 1958). Buck and his co-workers (1954; 1958) also consider the deposition of lipids (in cholesterol-fed rabbits) to be facilitated by preceding mucopolysaccharide accumulations in affected tissues.

Moreover, it can be seen from Fig. 6 that the rise in aortic calcium was accompanied by a large increase in the phosphorus content. This is of interest in view of the recent finding by Selye (1958) that administration of mono-sodium phosphate sensitizes the rat aorta to the calcifying effect of dihydrotachysterol. Selye's results, together with our observations, indicate that phosphorus is apparently an essential requirement for aortic mineralisation under these conditions, *i.e.*, calciferol and dihydrotachysterol intoxication.

The maintained serum mucoprotein elevations in our rats may perhaps also reflect the persistence of some profound alteration in connective tissue metabolism induced even by a single short period of calciferol intoxication. Whether such persistent alterations in connective tissue metabolism were due to some direct initial action of the calciferol itself, or to indirect effects consequent on early renal injury, as suggested by Gillman and Gilbert (1956*b*), or to secondary involvement of various endocrine glands, still remains problematical.

Relatively well established by our studies, however, is that aortic, coronary and other lesions once initiated, even by a single insult, are progressive and culminate in medial sclerosis, cartilage and even bone formation in the aortic wall. The heavy accumulation of MPS in the injured media of the coronary, renal and other medium-sized arteries studied by us, seems to be closely bound up with the

persistence and progress of the initial lesions. It is our impression, based on both microscopic and chemical data, that a new set of connective tissue regulatory mechanisms are initiated after the single period of calciferol intoxication. It is to this new set of regulations that both the progressive connective tissue MPS increases and the elevated SMP levels are perhaps due. The basic nature of the physiological and tissue enzymic changes still require study. However, it seems that the presence and even the degree of activity of reparative and degeneratory processes in connective tissue, particularly in arteries, may perhaps be predicted from the level of circulating SMP. For with the supervention of vascular collagenisation, later in our experiments, the SMP levels dropped (compare Figs. 4, 7 and 8).

The study of this aspect of the problem should indeed be rewarding. Our own analyses of the SMP in pregnancy and the puerperium in African women (Gillman and Pillay, 1959) and in the baboon (Gillman, Pillay and Naidoo, 1959) add weight to the possibility that active resolution or involutionary changes, at least in the connective tissues of the female genital tract, are closely associated with elevations in SMP levels (Gillman and Pillay, 1959). Moreover, Schwartz and Gilmore (1958) stress the ease with which SMP levels can be raised when depolymerisation of connective tissue ground substance is induced even by a single hyaluronidase injection.

We anticipate that increases in connective tissue MPS content will yet be shown to be closely associated with profound interference with the regeneratory processes in connective tissue, such as we have shown to be the case in lathyrus intoxicated rapidly growing weanling rats (Gillman and Hathorn, 1958 ; 1959).

Finally, we should like to stress, as we have before, (Gillman *et al.*, 1957) that, in agreement with Gillman and Gilbert (1956) there are obviously local peculiarities in the metabolic activity of the connective tissues in different sections of the arterial tree which make vessels vary in their susceptibility to the injurious actions of large doses of calciferol. Only on such a basis can we explain the obvious wave of calcification and decalcification (or persistent calcium deposits) detected in the heart, coronaries, aorta, etc. encountered at different times after administering calciferol.

The presently recorded chemically determined sequence of events indicates that healing in blood vessels is comparable only in broad trends with that noted by others in healing cutaneous wounds (Dunphy *et al.*, 1956 ; Jackson, 1957). All are now agreed that, in cutaneous wounds, there is initially an increase in hexosamine and that only later is this followed by a progressive increase in the amount of collagen. Repair of aortic injuries seems to follow a similar but not identical course.

However, there are at least two important differences between aortic and cutaneous repairs. Thus, in cutaneous wounds, hexosamine increase is notable within 2–4 days of injury, a decrease following in a further 2–3 days ; increases in chemically determined collagen have been recorded from the 4th day onwards. However, the hexosamine content of the aorta rises a little only 10 days after the medial injuries have been inflicted, remains elevated thereafter, but shows an even further increase at 160 days after the injury, possibly in association with both cartilage and additional collagen accumulation in injured areas. Collagen increase is always far less in the aorta than in skin wounds and becomes impressive very much later (Fig. 30), rather than in 2–3 days as is the case for the skin. The very

much slower rate of fibrosis as well as delayed hexosamine and cartilage accumulation in traumatised aortic tissue has been fully documented histologically in several previous studies (Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1959 ; Gillman, 1959*a*).

We have stressed some of the possible implications of these observations elsewhere, for we regard them as basically important for understanding the pathogenesis of arterial diseases (Gillman, 1957 ; 1958 ; 1959*a* ; Gillman *et al.*, 1957 ; Gillman and Hathorn, 1957 ; 1958 ; 1959). Simply stated, it seems justifiable to suggest that intramural scar formation, subsequent endarterial fibrin deposits and mural thrombosis, with ensuing vascular occlusion, may, at least in some instances be only the very late end results of even single incidents of vascular trauma, inflicted very much earlier in the life of the individual. Our earlier experimental data (Gillman *et al.*, 1957 ; Gillman, 1957, 1958 ; Gillman and Naidoo, 1957) indicating that healing acute vascular injuries may facilitate lipoidal and other degeneratory changes in blood vessels, have also been fully substantiated recently (Constantinides *et al.*, 1958).

We have also adduced evidence to indicate that two separate types of arterial sclerosis probably occur in man. The first type may originate in the manner just outlined above, namely, the sclerosis ensuing even many years after the infliction of acute injuries to arteries. As suggested elsewhere such " injuries " may be initiated by physical or metabolic " traumata " and, especially during that period of active aortic remodelling which is probably essential for the growth of the vascular system accompanying a rapid total body growth characteristic of adolescence (Gillman and Hathorn, 1959 ; Gillman, 1959*a* ; 1959*b* ; Gillman and Hathorn, 1958).

This type of vascular scarring may perhaps be compared with Harris' lines in bones, the latter being attributed to disturbances of osseous growth and remodelling caused by intermittent illnesses in young actively growing individuals. If rapidly growing bone may be damaged (" scarred ") by intermittent illnesses, it does not seem unreasonable to propose that rapidly growing vessels may likewise be susceptible, even though such injuries to arteries are detected only much later on in life when they become manifest in many ways. For the effects of repair of such vascular injuries, if they are (as we suspect) inflicted during adolescence, would not be detectable until they had progressed sufficiently to cause functional circulatory impairment.

The recent epidemiological studies of Holman *et al.*, (1958) would seem to substantiate the above orientation originally proposed by us more than a year ago (Gillman, *et al.*, 1957 ; Gillman and Hathorn, 1957 ; Gillman, 1957 ; 1958). For, Holman *et al.*, (1958) confirmed, on a grand scale, the basic observations of Moon (1957) that the earliest stages of arterial degeneratory change are frequently present in the aortae of pre-pubertal and adolescent individuals. However, Holman and his associates stated that fibrotic plaques were not notable in the aortae of either Europeans or Negroes until 10 to 15 years after the first lesions were detectable, *i.e.* only in individuals aged 30–35 years.

These findings in man are in direct conformity with our previously recorded experimental facts and the hypothesis based thereon (Gillman, 1957 ; 1958 ; Gillman and Hathorn, 1957 ; 1958 ; 1959). This type of " post-traumatic " arterial fibrosis may indeed account for the frequency of fibrosing arterial degeneratory disease in relatively young adults. If rapidly progressive, and especially if

aggravated by dietary and metabolic factors—and particularly those determined by sex hormones (Gillman, 1958 ; Gillman and Naidoo, 1958 ; Gillman, Naidoo and Hathorn, 1958)—then such early lesions may perhaps explain the great frequency of advanced coronary and other vascular lesions in certain individuals during the fourth decade of life. The recent experimental findings of Constantinides and co-workers (1958) on the facilitation of lipoidosis and thrombosis by previous injury of vessels, also completely support our previously expressed views.

The second type of vascular sclerosis which we think occurs, is that which results from the gradual deterioration of the regenerative processes in vascular tissues into repair processes. For, as described elsewhere (Gillman, 1958 ; 1959*a*, *b*) sclerosis (scar formation) does not represent connective tissue regeneration, but rather that type of connective tissue neogenesis attendant on tissue destruction with the ensuing architectural distortion inevitably associated with scar formation (Gillman, 1959*b*). Such sclerosis may supervene when, with ageing or through disease, the normal mechanisms for regeneration of " wear and tear " changes in tissues fail. While this type of vascular sclerosis may perhaps be initiated at any time in life, we consider it to be probably most prominent in biologically aged or ageing systems (Gillman, 1959*a*, *b*). The vascular sclerosis found in those individuals who have lived to a ripe old age probably represents a combination of both of the above types of arterial repair (Gillman, 1958 ; 1959*b*).

SUMMARY

The object of this study was to determine the histological, histochemical and chemical changes induced in the rat aorta by toxic doses of calciferol, and to compare these with changes found histochemically in the heart muscle and coronary arteries. To this end the hexose, hexosamine, collagen, elastin, calcium and phosphorus contents of the aorta, and the serum calcium and mucoprotein levels were determined, in a total of 90 rats at intervals up to 160 days after a 5-day period of calciferol administration. There was an equal number of pair-fed control rats.

The mineralization (13–14 per cent Ca^{++}) induced in the aorta by calciferol persisted for a long period, and was not related to the serum calcium level, which dropped rapidly after calciferol administration was discontinued. This persistence of aortic mineralization contrasted markedly with the disappearance about 10 days after the completion of calciferol administration of histochemical evidence of mineralisation of the heart and coronary arteries.

Chemically, the laying down of calcium in the aorta was closely paralleled by a large increase in the phosphorus content.

There was a prolonged rise in the serum mucoprotein level which contrasted sharply with the transient rise in serum calcium.

Histochemically, there was a general increase in periodic-acid Schiff positive material in calcified tissues. Chemically there was a rise in total aortic hexose and hexosamine to approximately double the control levels ; these increases, however, were not quantitatively related to the rise in calcium content of the aorta.

The total aortic collagen appeared to increase towards the end of the experimental period, coinciding with the histological appearance of aortic and coronary

sclerosis. This supports the concept that healing in vascular wounds pursues a much slower course than cutaneous wound healing.

The implications of these findings for the understanding of the pathogenesis of atherosclerosis and vascular regeneration, degeneration and repair are discussed.

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